

Full Length Research Paper

Isolation and characterization of efficient cellulolytic fungi from degraded wood and industrial samples

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Cellulose is the most abundant biopolymer and renewable natural product in the biosphere. Cellulose degrading fungal strains play an important role in recycling of cellulosic materials. They have immense advantage in various industries to hydrolyze cellulosic substrates for production of various products. This study was thus aimed to isolate and characterize efficient cellulose degrading fungi from their common natural habitats. Decaying Acacia wood and industrial water effluent samples were used for isolation and screening of cellulolytic fungi. Both samples were serially diluted and cultured on cellulose basal medium (CBM) supplemented with 30 mg/L chloramphenicol as bactericidal agent. Cellulose degrading fungal isolates were selected based on their hydrolyzed zone after congo red dye stain. Among 13 initial isolates, four isolates (C, E, G, and H) were finally screened as the most efficient fungal isolates representing only degraded Acacia tree. These isolates were confirmed as *Penicillium* species (C), *Apergillus terrus* (G), *Alternaria* species (H) and *Apergillus* species (E). From this study, the decaying Acacia sample was found to be the best source for cellulolytic fungi than that of wastewater sample. Out of these isolates, the maximum zone of hydrolysis (51.33±1.53 mm) was obtained for 'isolate E', whereas the minimum zone of clearance (26.67±1.53 mm) was recorded for *penicillium* species. This study indicates the existence of potential cellulolytic fungal on decayed wood of Acacia. Hence, further molecular aided characterizations of the isolates and their enzymes are of paramount importance for their use for industrial purposes.

Key words: Cellulase, cellulosic basal medium, congored, fungi, zone of hydrolysis.

INTRODUCTION

Cellulose is the most abundant polysaccharide that has tremendous economic importance around the globe. It is

a linear, unbranched homopolysaccharide consisting of glucose subunit joined by β 1-4 glycosidic linkage. It is a

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primary structural component of the plant cell wall accounting for over half of the carbon in the biosphere (Saha et al., 2006; Saranraj et al., 2012). Some of cellulosic biomass includes agricultural and forestry residues, municipal solid waste, industrial wastes, and herbaceous and woody plants (Saranraj et al., 2012). Cellulose is an important material from which industrially valuable products are produced. Bisaria and Ghose (1981) reported the substantial economic interest to develop processes for effective treatment and utilization of cellulosic waste as inexpensive carbon sources. Enormous amount of agricultural, industrial, and municipal cellulosic wastes have been accumulating and used inefficiently due to the high cost of their utilization process. However, cellulose is important raw material for energy production and to minimize load of wastes and greenhouse effect (Yue and Wenchin, 2008). Plant biomass is major source of fuels; animal feeds, and feedstock as renewable and alternative energy source (Bhat, 2000). These make the biotransformation of cellulose, an important part of energy recycling in biosphere which can be achieved with the help of cellulases.

The study indicates that the cost of ethanol production from cellulosic material using chemical method is considerably high (1.8 USD/gallon). Production of industrial products using chemical method is not only costly, but also hampers the ecosystem. The development of enzymatic processing can decrease the cost of ethanol to about 0.2 USD/gallon (Genansounou, 2010). This high industrial cost of production can be decreased with the use of microbial cellulases, which is economical when compared to other chemical means of production. Cellulases have enormous potential in industrial application. Glucose produced from cellulosic substrate by hydrolysis could be further used as substrate for subsequent fermentation or other process which could yield valuable end products like ethanol, butanol, methanol, amino acid, single cell protein and organic acid (Walsh, 2002). Cellulases are a group of hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components that can later be converted to various industrial products. Cellulolytic enzymes also play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded (Peciulyte, 2007). Cellulase is multiunit enzyme composed of several enzymes with numerous isozymes (Siddiqui et al., 2000). It comprises three classes of soluble extra cellular enzyme: 1, 4- β -endoglucanase, 1, 4- β exo-glucanase, and β -glucosidase or cellobiase (Siddiqui et al., 2000, Nishida et al., 2007). Endoglucanase hydrolysis internal beta -1,4-glucan chain of glucose, Exo-glucanase remove cellobiose from the non-reducing end of cello-oligosaccharide, cellobiase hydrolysis cellobiose to yield two molecules of glucose which deplete the depolymerization of cellulose.

Biotechnological conversion of cellulosic biomass is

potentially sustainable approach to develop novel bioprocesses and products. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications (Kuhad et al., 2011). The search for efficient microorganisms which can produce all the three types of cellulases that can facilitate the breakdown of cellulose to glucose is of paramount importance (Maki et al., 2009). There has been much research aimed at obtaining new micro-organisms producing cellulase enzymes with higher specific activities and greater efficiency (Rathnan et al., 2012). Fungi and bacteria are the main agent of cellulosic degradation (Lederberg, 1992). The cellulose utilizing population of microorganisms includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, *Actinomycetes* and certain protozoa (Alexander, 1961). Among them, the genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulase producer (Sukumaran et al., 2005; Kuhad et al., 2011). Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd et al., 2002; Peciulyte, 2007). Although a large number of fungi can degrade cellulose, only a few of them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose (Rathnan et al., 2012).

Fungal cellulases are less complex than that of bacterial origin and they can be more rapidly cloned and produced (Maki et al., 2009). Nowadays, there is a great concern of obtaining new microorganisms producing cellulolytic enzymes with higher specific activities and greater efficiency (Johnvesly et al., 2002). Considering the importance and application of the fungal cellulases, this study was designed to isolate and characterize efficient cellulose degrading fungi from decayed Acacia wood and water samples of industrial area to lay a base for industrial application of cellulase producing fungi.

MATERIALS AND METHODS

Sample collection and processing

Potential samples containing cellulolytic fungi were collected from degraded Acacia tree in university of Gondar, Maraki campus and wastewater treatment pond of Dashen brewery industry, Gondar city, Ethiopia. Wood sample was collected in sterile plastic bag and stored at -20°C until use. The water sample of industrial effluent was also collected using sterile baby jar and stored at -20°C. Serial dilution of wood samples was made by adding 1 g of degraded wood in 9 mL of distilled autoclaved water. For industrial water samples, 1 mL was taken and serially diluted in the distilled autoclaved water.

Culture media preparation and initial culturing

Three types of culture media were used for this study; Cellulose Basal Medium (CBM) with the following composition in (g/L):

Table 1. Total colonies recovered on selective cellulosic basal media after five days of incubation.

Sample	Dilution number	Number of colonies with visible cellulolytic activity	Feature of colony on PDA
Wood	10^{-2}	6 = (A, B, C, D, E, F)	Olive green with reverse pale yellow (A-D) Fuzzy black, reverse white (E and F)
	10^{-4}	2 = (G, H)	Cinnamon to brown, reverse white (G) Ocher, reverse black (H)
Industrial water	10^{-3}	3 = (HA, HB, HC)	Gray green (HA) White (HB and HC)
	10^{-5}	2 = (HD and HE)	Both are white

NH_4NO_3 ; 2, KCl; 0.5, KH_2PO_4 ; 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01, cellulose; 15, agar-bacteriological; 20, and penton; 0.2 was used for initial culturing and further plate screening. Additionally, the CBM was supplemented with 30 mg/L of chloramphenicol to inhibit the growth of bacteria. For sub-culturing and morphological based identification of screened potential cellulolytic fungi, potato dextrose agar (PDA) supplemented with 30 mg/L of chloramphenicol was used. Finally, trypto soya broth was used for preservation of pure isolates for further analysis. Appropriate constituents of all media were measured, dissolved, pH set to 5, and autoclaved properly. In all cases, 30 mg/L of chloramphenicol was added after autoclaving in sterile condition under the hood. The medium was then transferred to sterile Petri-dishes and allowed for solidification. About 0.1 mL of serially diluted suspension of both samples was spread onto solidified CBM and incubated at 30°C for four days.

Plate screening and subculture of cellulolytic fungi

After four days of incubation at 30°C, colonies grown on CBM petriplates with dilution number of 10^{-2} to 10^{-5} were flooded with 0.1% Congo-red dye. The diameter of clearance around colony was taken as reference to further screen isolated colonies based on their efficiency on CBM. The colonies with relatively higher zone of clearance were transferred to another freshly prepared CBM. After four days of incubation, it was again flooded with 0.1% Congo-red dye and the zone of clearance was measured. Highly efficient cellulolytic fungal isolates were selected on the basis of the diameter of the hydrolysis surrounding the colonies. All colonies with higher clearing zone were taken and preserved in trypto soya broth at 4°C for macroscopic and microscopic identification.

Measurement of cellulolytic activity from CBM well diffusion

Chloramphenical containing Cellulosic Basal Medium (CBM) was prepared and solidified at room temperature. A well with about 6 mm diameter was made on each solidified medium with the help of borer. About 50 μL of preserved isolates was then added to each bore in triplicate and incubated for six days at 30°C in light preventing incubator. Petri plates were then flooded with 0.1% Congo red solution, left for 15 min with intermittent shaking in a mechanical shaker, and washed with distilled water two to three times to remove unbound congo red dye. Finally, the plates were washed with 1 M NaCl solution and the zone of hydrolysis was measured for all replicates and the mean of all replicates was then recorded.

Identification and characterization of efficient fungal isolates

In order to identify and characterize the fungal colonies, both morphological characteristics on Potato Dextrose Agar (PDA) and microscopic examination was recorded. Only isolates with relatively higher zone of clearance was selected and streak plated on PDA, and incubated at 30°C. The colonies were inspected each day for any change in color, speed of growth, and growth pattern. Colony color, shape, border and spots were recorded. The microscopic study was conducted using lacto-phenol cotton blue stain. A drop of lacto-phenol cotton blue solution was added on a slide and four days old fungal culture was put on microscopic slide, mixed with lacto-phenol stain, covered with the clean cover slip, and microscopically observed under high power oil immersion objective (100X). Spores and the mycelia were observed and the data were recorded and used for classification as described in Domsch et al. (1980).

Data analysis

Data were recorded, checked for completeness and consistency and subjected to analysis using Excel and SPSS version 16.0 software packages. One-way ANOVA was used for multiple mean comparisons (Student-Newman-Keuls). $P < 0.05$ was considered significant during the analysis.

RESULTS

Primary selection and screening of potential cellulolytic fungi

After five days of initial culture on selective CBM, 13 colonies (six colony from wood samples with dilution 10^{-2} , two colonies from wood sample dilution 10^{-4} , three colonies from industrial samples with dilution number 10^{-3} and two colonies from industrial samples with dilution number of 10^{-5}) having better hydrolysis zone were selected (Table 1). From these initial isolates, four colonies of wood samples (labeled as A, B, C, and D) recovered from dilution number 10^{-2} were morphologically similar. However, colony 'C' showed higher zone of hydrolysis (26.67 mm) on CBM agar.

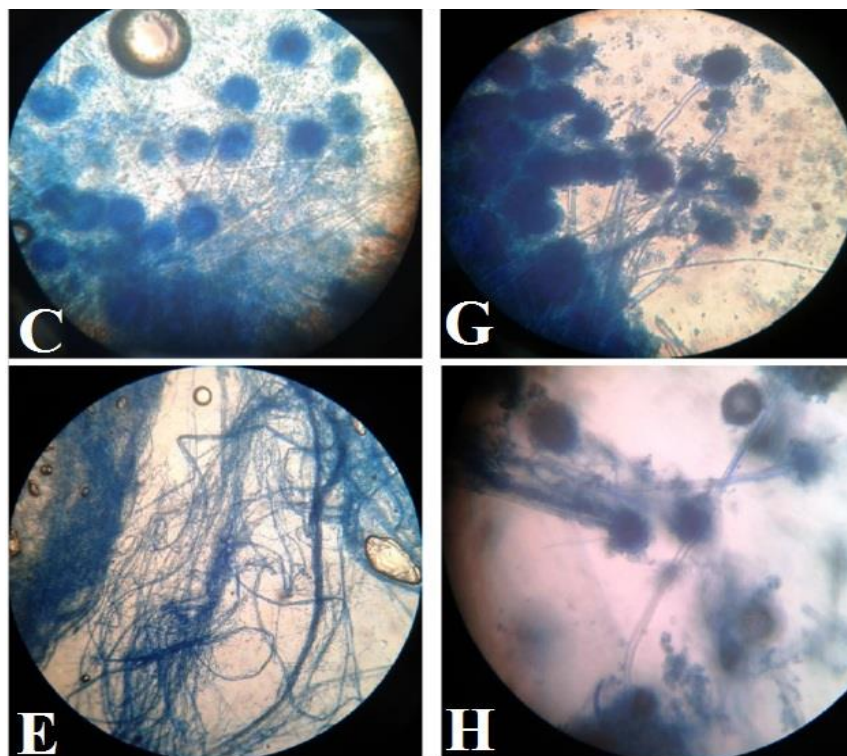


Figure 1. Microscopic features of four efficient isolates. C = *penicillium* spp.; G = *Aspergillums terreus*; E = not confirmed; H = *Alternaria* spp.

Sub-culture and secondary screening

All primarily isolated 13 colonies were then again sub-cultured on selective CBM and incubated again for five days. The zone of hydrolysis was then measured for all isolates and the most efficient isolates in terms of zone of clearance were selected for further analysis. The most efficient fungal isolates were isolate C and E from wood samples of dilution number 10^{-2} and G and H from wood samples of dilution number 10^{-4} . Two isolates of industrial samples (HA and HB) failed to show better zone of hydrolysis as compared to woody samples. Isolate HA was white and grow fast with low zone of clearance on CBM agar. Hence, both water samples were not further studied as their zone of hydrolysis was very lower.

Morphological and microscopic results of efficient isolates

From the thirteen primary isolates, only four isolates (C, E, G, and H) were screened for morphological and microscopic identification as they showed relatively wider zone of hydrolysis due to the release of exo-cellulose. Isolate 'C' was initially white and become yellowish, gray green with yellow reverse. The microscopic examination indicated that it has round and unbranching conidia at the

tips of the phialides with septate hyaline hyphae (Figure 1). It was then identified *aspericillium* species. In the same manner, the isolate G and H were identified to be *Aspergillums terreus* and *Alternaria* species. However, isolate 'E' was not yet identified to species level due to the absence of strong evidence from microscopic data. Presumably, it was *Aspergillus niger* with dark green color on frontal part of PDA and unpigmented (white) reverse (Table 2).

Cellulolytic activity of the four efficient isolates

From the measurement of zone of hydrolysis, the maximum zone of hydrolysis was obtained for 'isolate 'E' (51.33 mm). The lowest zone of hydrolysis (26.67 mm) was recorded for *penicillium* spp. (isolate 'C'). Of all the isolates, significantly high zone of hydrolysis ($P < 0.05$) was obtained for isolate 'E' (*Aspergillums* species) but there is no significant difference of zone of hydrolysis ($P = 0.11$) between isolates 'C' and 'G' (Table 3; Figure 2).

DISCUSSION

Being the most abundant organic substance on the earth (Ruttloff, 1987), cellulose comprises photosynthesized

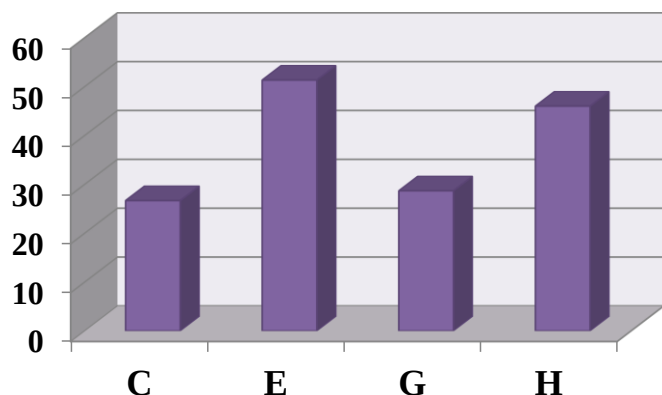
Table 2. Morphological and microscopic characteristic of efficient fungal isolates.

Isolate	Morphological features	Microscopic characteristics	Identified fungal species
C	Colonies are initially white and become gray green after 7 days. The reverse is yellowish	The conidia are round and unbranching at the tips of the phialides, septate hyaline hyphae	<i>Penicillium</i>
E	Dark green to black. The reverse is white	Grassy, no hyphae and other fruiting bodies	<i>Aspergillums</i> species
G	Cinnamon to brown, reverse white	Hyphae are septate and hyaline	<i>Aspergillums terrus</i>
H	Ocher, reverse black	Septate hyphae,	<i>Alternaria</i>

Table 3. Mean diameter of hydrolysis of all efficient isolates on CBM.

Isolate	Zone of hydrolysis (mm)			Mean (mm)	SD
	ZH1*	ZH2	ZH3		
C	25	26	29	26.67 ^a	1.53
E	50	53	51	51.33 ^c	1.53
G	28	28	30	28.67 ^a	1.16
H	44	46	48	46 ^b	2.00

*ZH=Zone of hydrolysis; SD=Standard deviation. Means with identical alphabetic letters are not significantly different at p =0.05.

**Figure 2.** Mean zone of clearance (mm) of four isolated efficient fungal types.

glucose. It is the major component of biomass energy (Scott et al., 1987). Fungi are well known agents of decomposition of organic matter in general and of cellulosic substrate in particular (Lynd et al., 2002). Fungal cellulases have great application in wood and starch, paper, and textile industries (Saranraj et al., 2012). Maki et al. (2009) reported that fungal cellulases are less complex and efficient than bacterial counterparts. In this study, partially decayed Acacia tree and waste water treatment pond of brewery industry that supposed to be natural habitat for cellulolytic fungi were utilized for efficient fungal isolation. After several

screening of fungal isolates of both samples, four efficient isolates (C, E, G and H) found to possess relatively higher zone of inhibition were screened and identified. The isolates were then characterized as *Penicillium* species (C), *A. terrus* (G), *Alternaria* species (H) and isolate 'E' (*Aspergillus* species). All of this four isolates were recovered from decayed woody samples of Acacia tree. This might be due to the fact that Acacia is best habitat for growth of such microbial system since the peel of Acacia tree has very good nutrition for primary attachment. Similar to our study, Lakshmi and Narasimha (2012) identified potential cellulolytic fungal isolates from forest litter. Efficient cellulolytic fungi were also isolated from natural resources by Rathnan et al. (2012). Highly efficient *Aspergillus* species were also isolated from plant (Oliveira et al., 2012). All of industrial samples were not selected for cellulolytic activities due to lack of good zone of hydrolysis. The absence of efficient cellulolytic fungi from such samples might be due to lack of good nutrition, toxic effects, habitat competition in water body with other group of microorganisms and the chemical they uses might inhibit the growth of cellulolytic fungal species. Lekhrum et al. (2014) reported low zone of hydrolysis with fungal strain isolated from soil. Low cellulolytic activities of soil isolated fungi was also reported in other studies when compared with wood and wood related sample isolates (Ram et al., 2014).

Various studies indicated that majority of *Aspergillus*, *Fusarium*, *Alternaria*, *Rhizopus*, *Penicillium* and *Trichoderma* isolates were found to possess cellulolytic

activity (Vries and Visser, 2001; Gunathilake et al., 2013). As stated in Yalpani (1987) the most common and most potent cellulase producers are *Trichoderma reesei*, *Trichoderma koningii*, *Fusarium*, *Aspergillus* and *Penicillium* sp. In present study, out of the four efficient isolates screened, *Aspergillus* species showed greater hydrolysis zone (51.33 mm). Similar to our study, Jahangeer et al. (2005) indicated that majority of *Aspergillus* and *Penicillium* species were found to possess higher cellulolytic activity. However, *Penicillium* species are inferior to three of the present isolates. According to Mirzaakhmedov et al. (2007), *A. terreus* was the most active producer of cellulolytic enzymes than *Penicillium*, *Fusarium*, *Trichoderma* and other *Aspergillus* species. The present study also indicated that *A. terreus* was better than *Penicillium* species, though inferior to Isolate 'E' and *Alternaria* species. Our study also supported by the study of Lakshmi and Narasimha (2012) that recovered potential *Aspergillus* species with maximum zone of hydrolysis (42 mm) than *Penicillium* species. The study of efficient cellulolytic fungi isolation from natural resources by Rathnan et al. (2012) also reported 44 mm as maximum zone of hydrolysis which is closer to our finding (51.33 mm).

Conclusion

In this study, 13 primary fungal isolates were grown on CBM. Out of the 13 isolates only four efficient isolates namely: *Penicillium*, *A. terreus*, *Alternaria* and isolate E (*Aspergillus* species) were recovered. *Aspergillus* species (isolate E) showed maximum zone of hydrolysis followed by *Alternaria* species, *Penicillium* and *A. terreus*. In this study, the decaying Acacia tree was found to be a good source of cellulolytic fungi species. For application in industries, further characterization and optimization of the culture condition is needed to suite industrial application of this potential fungi species for cellulose bioconversion in industries.

Conflict of interests

The authors did not declare any conflict of interest.

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